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Genetic Complexity of the Hypervariable Region 1 (HVR1) of Hepatitis C Virus (HCV): Influence on the Characteristics of the Infection and Responses to Interferon Alfa Therapy in Patients With Chronic Hepatitis C

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HCV exists within its host as pools of related genetic variants referred to as quasispecies. The hypervariable region 1 (HVR1) of the E2 envelope gene is subjected to strong selective pressure from neutralizing antibodies. The genetic complexity of this region is defined as the total number of genetic variants within the quasispecies population. The genetic complexity of the HVR1 region was examined in patients with chronic hepatitis C and its relationship with the epidemiology of HCV infection, and its influence on liver disease and the response to interferon treatment were determined in 114 patients with chronic hepatitis C. The genetic complexity of the HVR1 major variants was measured before treatment by using a polymerase chain reaction (PCR)-single-strand conformation polymorphism (SSCP) technique, and was compared with epidemiological, clinical, virological and histological features. The patients were treated with 3 megaunits of interferon (IFN) alfa for 3 to 6 months and the response to treatment was assessed at 3, 6 and 12 months. The HVR1 could be studied in 101 of the 114 patients (89%). Genetic complexity was significantly higher in patients infected through blood transfusion than intravenous drug use (mean complexity index: 5.7 ± 2.3 vs. 4.7 ± 1.5, respectively; $P = 0.04$). This relationship was independent of age and the estimated time since infection. No significant relationship was found with other parameters of infection or liver disease. In univariate analysis, the genetic complexity of HVR1 major variants did not affect the rates of ALT normalization at months 3 and 6 of IFN treatment. HVR1 genetic complexity was lower in patients with a sustained virological response than in non-responders (4.0 ± 1.7 vs. 5.4 ± 2.0, respectively; $P = 0.07$). In multivariate analysis of pretreatment parameters associated with a sustained virological response to treatment, three parameters appeared to be independent predictors of such a response: a low viral load ($P < 0.04$), a low anti-HCV core IgM titer ($P = 0.03$) and a low genetic complexity of HVR1 major variants ($P < 0.04$). In conclusion, the HVR1 of HCV has a quasispecies distribution in infected individuals. Its genetic complexity is significantly higher in transfusion recipients than in intravenous drug users, suggesting that the size of the initial inoculum affects the later emergence and development of viral quasispecies. The genetic complexity of HVR1, together with viral load and the anti-HCV IgM titer, are independent predictors of a sustained virological response to IFN alfa in patients with chronic hepatitis C. ***J. Med. Virol. 54:256–264, 1998.***

KEY WORDS: hepatitis C virus; hypervariable region 1; quasispecies; genetic complexity; interferon alpha

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INTRODUCTION

Hepatitis C virus (HCV) is responsible for most cases of non-A, non-B hepatitis worldwide [Alter et al., 1989; Hopf et al., 1990]. After the acute phase of infection, at least 80% of patients develop chronic hepatitis C. HCV infection appears to be pleomorphic, with a wide range of disease severity and natural history [Alter 1995], clinical manifestations (hepatic and extra-hepatic involvement) [Alter 1995; Pawlotsky et al., 1995a; Pawlotsky et al., 1995b], and sensitivity to treatment, which is currently based on interferon (IFN) alfa [Hoofnagle et al., 1986; Mahaney et al., 1994; Martinot–Peignoux et al., 1995; Pawlotsky et al., 1996b]. The recently demonstrated "quasispecies" nature of HCV genome distribution [Martell et al., 1992], which probably results from complex interactions between the host and the infecting viral strains, has been forwarded to explain both the high rate of viral persistence and the wide range of disease manifestations and outcomes.

HCV, like other RNA viruses [Eigen and Biebricher, 1988; Holland et al., 1992], exists within its host as pools of related genetic variants referred to as quasispecies. This confers a significant survival advantage because the simultaneous presence of multiple variant genomes and the high rate of generation of new variants allows for the rapid selection of the mutants with better fitness for any new environmental condition [Martell et al., 1992]. The genetic heterogeneity within the HCV quasispecies population results from a high RNA-dependent RNA polymerase error rate (with misincorporation frequencies averaging about 10^{-4} to 10^{-5} per base site), and the apparent absence of any error correction of proofreading mechanism [Clarke et al., 1994]. Most mutant viral particles cannot replicate, but the remainder can transmit new genetic information to their progeny. The fittest infectious particles are selected by their replication capacities and especially by the selective pressure exerted by host cells and the immune response (the so-called "genetic bottleneck" [Duarte et al., 1994]). Immune selective pressures act on regions encoding cytotoxic and neutralizing epitopes. The hypervariable region 1 (HVR1) of the genome is located at the 5′ end of the E2 envelope gene and encodes a 27 amino acid stretch which is a target for the anti-HCV neutralizing response [Weiner et al., 1992; Farci et al., 1994]. It is commonly used to maximize the detection of quasispecies variants.

Whatever the region studied, two components of quasispecies heterogeneity must be distinguished: "genetic complexity" has been defined as the total number of genetic variants within a quasispecies population, while "genetic diversity" has been defined as the average distance between variants in a quasispecies population [Gretch and Polyak, 1997]. Recent data suggest that HCV RNA heterogeneity is involved in viral persistence, the pathogenesis of hepatic and extra-hepatic disease and the responses to IFN alfa therapy [Gretch and Polyak, 1997]. However, most studies suffer from incomplete definition of the parameters studied and comparisons are made difficult by differences in the methods used.

The aim of the present study was to determine, by means of a standardized and reproducible polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) technique: (i) the relationship between HVR1 genetic complexity and the epidemiology of HCV infection, (ii) the relationship between genetic complexity and the other features of HCV infection and the related liver disease, and (iii) the influence of pretreatment HVR1 genetic complexity on the biochemical and virological responses to IFN alfa.

MATERIALS AND METHODS

Patients

One hundred and fourteen consecutive patients with chronic hepatitis C (76 men, 38 women, mean age 46.2 ± 13.9 years, range 18 to 74) eligible for IFN therapy were studied. The inclusion and exclusion criteria have been described recently [Pawlotsky et al., 1996b]. The trial was approved by the institutional ethics committee and all the patients gave their written informed consent. Before treatment, HVR1 genetic complexity was studied in all 114 patients by means of a reverse transcriptase-PCR SSCP procedure developed in our laboratory. In addition, the following information was collected: age, gender, apparent source of infection (blood transfusion, intravenous drug use, or unknown), estimated duration of HCV infection in patients with an identified risk factor, serum ALT and gamma-glutamyl transpeptidase (γ-GT) activities, presence of cirrhosis on liver biopsy, HCV genotype, serum level of HCV RNA (viral burden) and titer of anti-HCV core IgM antibodies.

All 114 patients were treated with 3 megaunits of IFN alpha-2a (Roferon™-A, Roche Laboratories, Basel, Switzerland) subcutaneously three times a week for at least 3 months. After 3 months of therapy, patients with elevated ALT were considered non responders. Patients with normal ALT activity at month 3 all received a further 3 months of treatment at the same dose. Serum ALT activity was used as a biochemical index of the response to IFN: i) at month 3 (early biochemical response), ii) at month 6, i.e. when IFN was discontinued in all the patients (biochemical response at treatment withdrawal), and iii) at month 12, i.e. at least 6 months after treatment withdrawal. At the latter date a sustained biochemical response was defined as normal ALT activity. All patients with a sustained biochemical response at month 12 underwent HCV RNA screening by PCR. Patients who were PCR-negative at this time were defined as sustained virological responders.

Evaluation of HVR1 Genetic Complexity

A standardized RT PCR-SSCP method was developed in our laboratory to evaluate the overall genetic complexity of HCV strains in the serum of infected individuals [Orita et al., 1989; Sekiya, 1993]. Briefly, RNA was first extracted from 50 μl of serum with RNA-

zol (RNA-B, Bioprobe Systems, Montreuil-sous-Bois, France) and chloroform, and reverse transcribed at 42°C for 90 min by using 7 pmol of the downstream primer (5′GGTGTGGAGGGAGTCATTGCAGTT3′, nucleotide position 1611–1634 [Enomoto et al., 1994]) in the presence of 8 units of avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, Wisconsin). PCR was performed using 5 pmol each biotinylated downstream primer and upstream primer (5′GCTTGGGATATGATGATGAACTGGTC3′, nucleotide position 1284 to 1309 [Enomoto et al., 1994]) with 2.5 units of *Taq* DNA polymerase (Pharmacia Biotech, Uppsala, Sweden). After denaturation for 5 min at 94°C, PCR comprised 45 cycles (94°C, 1 min; 68°C, 1 min; 72°C, 1 min). Positive and negative controls were included in each series. Amplified products were analyzed by electrophoresis through 3% NuSieve agarose gel (FMC, Rockland, Maryland) and staining with ethidium bromide.

Amplified products were then extracted from the agarose gel and purified with the Sephaglas BandPrep Kit (Pharmacia Biotech) according to the manufacturer's instructions. Purified PCR products were eluted in 20 μl of distilled water. SSCP analysis was performed by using the PCR Fragment Analysis Kit (Pharmacia Biotech). An average of 50 ng of amplified DNA recovered from each serum sample was diluted in 4.5 μl of sterile distilled water and 4.5 μl of a solution containing 10 mM NaOH and 2 mM EDTA; bromophenol blue was then added. The samples were denatured for 10 min at 100°C and immediately chilled on ice. Eight microliters of the denatured samples was then loaded into the wells of a discontinuous polyacrylamide gel (CleanGel, Pharmacia Biotech) which had been rehydrated to a thickness of 0.5 mm with a buffer specially designed for DNA separation (pH 7.3). Horizontal electrophoresis was run in the Multiphor II electrophoresis apparatus (Pharmacia Biotech) at 9°C at 100 V for 20 min (penetration in the gel) and 600 V for 60 min (migration and stacking).

The gel was then submitted to a rapid and sensitive silver staining procedure using the Silver Staining Kit DNA (Pharmacia Biotech), a procedure that can detect 0.5 to 2 ng of DNA. After electrophoresis the gel was fixed for 30 min at room temperature in 10% acetic acid, washed and incubated for 30 min in 200 ml of a solution containing 0.1% $AgNO_3$ (w/v) and 0.1% formaldehyde. The gel was rinsed, placed in 200 ml of a solution containing 2.5% Na_2CO_3, 0.1% formaldehyde and 0.002% sodium-thiosulphate, and agitated slowly until staining became visible. The reaction was stopped by 20-min incubation in 10% acetic acid and staining was preserved by 20-min incubation at room temperature in a solution containing 5% glycine and 10% acetic acid.

The gels were scanned, and the band patterns were analyzed objectively on a computer by means of the ImageMaster 1D software (Pharmacia Biotech). The optical density of each band was determined and the bands with an optical density higher than 0.03 were taken into account in the analysis. The number of bands was calculated in each case and reflected the total number of HCV variants present in serum, i.e. the genetic complexity of the HCV genome.

To assess the sensitivity of the technique, we sequenced more than 30 clones originating from HVR1 PCR products from five patients. PCR products were purified using Sephaglas BandPrep kit (Pharmacia Biotech) according to the manufacturer's protocol. Purified products were quantified by ethidium bromide staining using DNA standards as controls and 50 ng was directly ligated into 50 ng pTAg vector (LigATor Cloning Kit, R & D Systems, Abingdon, UK). Transformation of recombinant plasmid DNA into *Escherichia coli* competent cells was performed according to manufacturer's protocol (R & D Systems) and transformants were grown on ampicillin-tetracyclin plates. The clones were sequenced with the AutoLoad™ Solid Phase Sequencing Kit used on the ALF™ Express automated DNA sequencer (Pharmacia Biotech). These experiments showed that our PCR-SSCP technique is capable of identifying point mutations and of separating and visualizing clones which represent more than 10% of the viral population in the quasispecies. Thus, by definition [Gretch and Polyak, 1997], the number of bands calculated for each patient with our technique reflects the genetic complexity of the quasispecies HVR1 "major variants".

HCV Genotyping

The Inno-LiPA HCV method (Innogenetics S.A., Gent, Belgium) [Stuyver et al., 1993] was used. The highly conserved 5′ non coding region of the HCV genome was amplified by a reverse transcriptase "nested" PCR using two sets of universal HCV primers. The amplified products were then hybridized to immobilized oligonucleotide probes specific for the different HCV genotypes and subtypes, as recently described [Pawlotsky et al., 1995c].

Detection of HCV RNA by PCR

To assess the virological response to treatment, HCV RNA was tested in the serum of patients with a sustained biochemical response to IFN therapy at month 12, by using a highly sensitive RT "nested" PCR procedure [Pawlotsky et al., 1994].

Quantification of Serum HCV RNA

HCV RNA was quantified in 50 μl of serum by means of the second-generation signal amplification assay based on branched DNA (bDNA) technology (Quantiplex™ HCV RNA 2.0, Chiron Diagnostics, Emeryville, California), according to the manufacturer's instructions [Urdea 1997]. In contrast to the initial assay, this version quantifies HCV RNA independently of the HCV genotype [Detmer et al., 1996]. The mean value of duplicate determinations, performed independently on each serum sample, was compared to a standard curve constructed in each run from standards containing known amounts of synthetic HCV RNA. The results

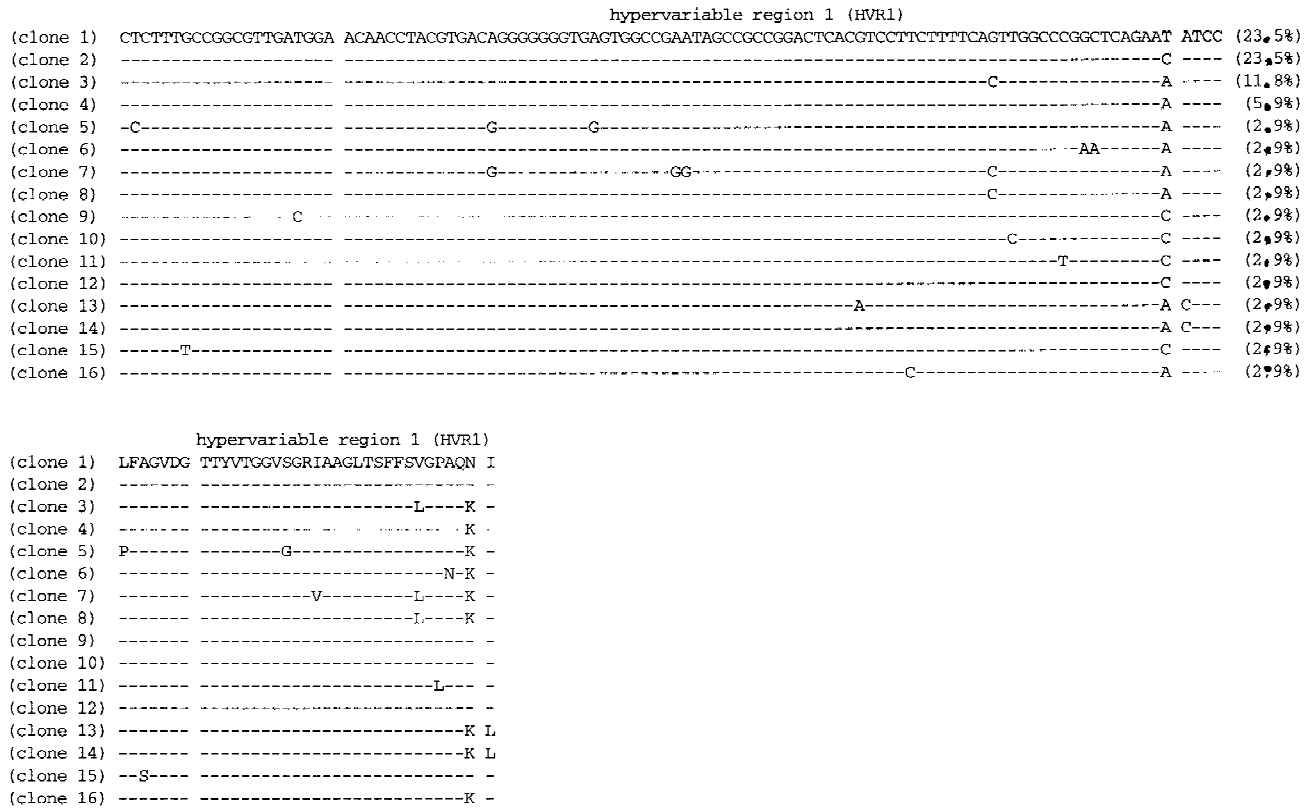

Fig. 1. Nucleotide and deduced amino acid sequences and frequency of the clones isolated from a patient from this study. The PCR-SSCP technique used in this study was able to discriminate between point mutations. Most of the mutations were within hypervariable region 1, but some occurred upstream and/or downstream. Some mutations were silent and did not induce amino acid changes.

were expressed in millions of genome equivalents per ml or megaequivalents per ml (MEq/ml). The manufacturer's cutoff is 0.2 MEq/ml.

Quantification of Anti-HCV Core IgM Antibodies

Anti-HCV core IgM was tested by the HCV IgM EIA 2.0 kit (Abbott Diagnostic, Chicago, Illinois) according to the manufacturer's protocol. An index value reflecting the amount of anti-HCV core IgM was calculated for each patient from the optical density of the sample, as recommended by the manufacturer.

Statistical Analysis

The results are presented as means ± 1 standard deviation, or as percentages. The non-parametric Mann and Whitney or Kruskall–Wallis tests were used to test quantitative variables. The Chi square test and, when necessary, Fisher's exact test, were used to test categorical variables. Univariate analysis was undertaken to determine whether the genetic complexity of HVR1 was associated with the response to IFN treatment. A multivariate analysis was then performed to identify factors associated independently with the response when genetic complexity was significantly associated with it in univariate analysis. In the stepwise logistic regression model, explanatory variables were those found to be significantly related to the response in the univariate model. The *P* value for entry had to be less than 0.10. Both univariate and multivariate analyses were performed using BMDP statistical software (BMDP Statistical Software Inc., Los Angeles, California).

RESULTS

Quasispecies Distribution Based on HVR1

To confirm the sensitivity of our technique in the study population, we cloned HVR1 PCR products from five patients into the pTAg vector and analyzed more than 30 clones per patient by PCR-SSCP. This procedure (clonal frequency analysis) allowed us to determine the frequency of each SSCP pattern, that is the frequency of each sequence in the quasispecies. Clones with different SSCP patterns were sequenced and, when a pattern was observed in more than one clone, 2 to 3 identical clones were sequenced. This procedure showed that the patients all harbored a complex mix of closely related variants, confirming the quasispecies distribution of HVR1 [Martell et al., 1992; Weiner et al., 1992]. The viral quasispecies comprised both major variants, representing more than 10% of the clones isolated, and minor variants, the sequences of which could be unique [Gretch and Polyak, 1997]. Figures 1 and 2 give the nucleotide and amino acid sequences of the

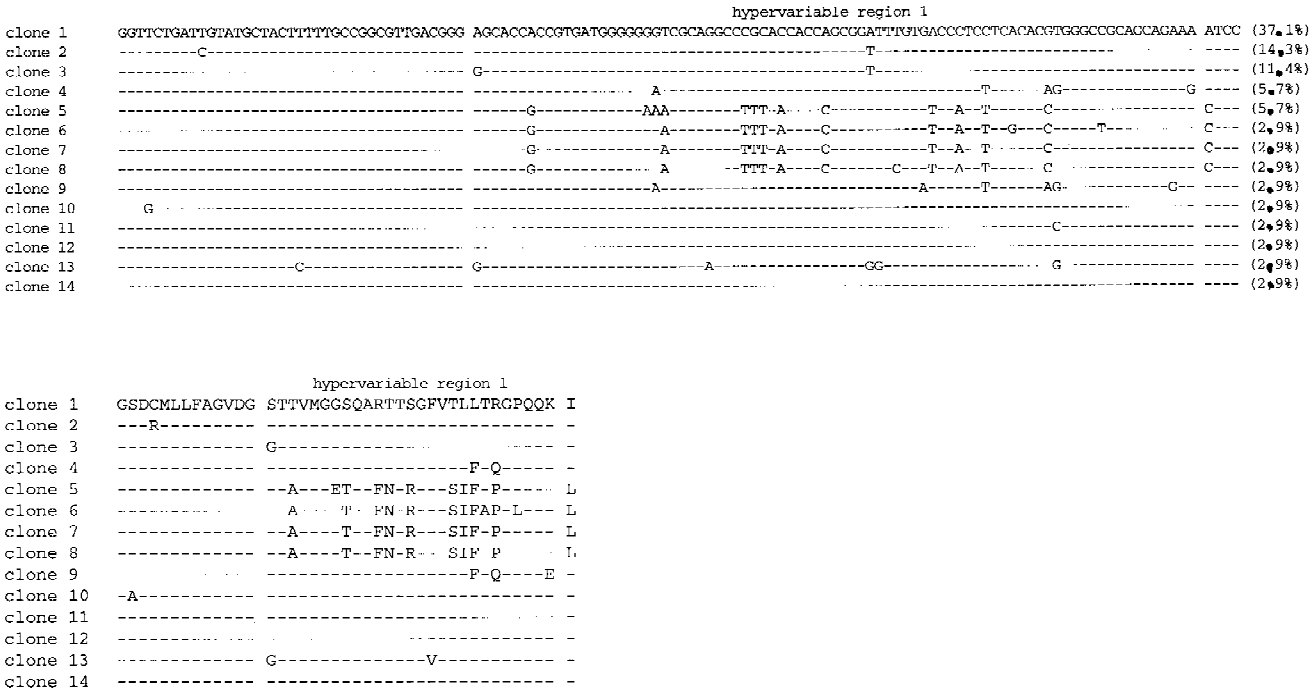

Fig. 2. Nucleotide and deduced amino acid sequences and frequency of the clones isolated from another patient from this study. The mutations characterizing clones 12 and 14 were upstream the shown sequence. Some mutations were silent and did not induce amino acid changes. In this patient, the genetic diversity was higher than in the patient in Figure 1.

clones and their relative frequencies in two of the patients. Both patients had close genetic complexities, but the genetic diversity was higher in the patient in Figure 2.

These findings indicated that our PCR-SSCP technique was capable of discriminating among viral populations differing by as little as point mutations. The relative positions of the bands in the gel in a defined patient were independent of the genetic distances between the corresponding viral variants. Finally, owing to the characteristics of the initial PCR amplification, the technique was capable of separating and visualizing viral populations that represent more than 10% of the viruses in the quasispecies. Thus, the number of bands calculated with our PCR-SSCP technique was an index of the genetic complexity of the quasispecies HVR1 major variants [Gretch and Polyak, 1997], a parameter which could be compared to the other features of HCV infection.

Genetic Complexity of HVR1 Major Variant

PCR amplification of HVR1 was possible in 101 of the 114 patients (89%) with the primers used, a sensitivity comparable to that observed when using primers designed in regions other than the 5′ untranslated one [Enomoto et al., 1994]. Negativity in the PCR assay was not associated with a particular genotype, nor with lower viral load. The 101 PCR products were analyzed by SSCP and the number of bands was calculated in each sample by using Image Master 1D software (Pharmacia Biotech), in order to assess the genetic complexity of the HCV major variants. In the 101 samples analyzed, the mean number of bands was 5.4 ± 2.0 (range 2 to 11).

Relationship between the Genetic Complexity of HCV Major Variants and Epidemiological, Clinical, Histological and Virological Characteristics of the Infection

The results are presented in Table I. Genetic complexity was significantly related to the route of HCV transmission. Indeed, genetic complexity measured by PCR-SSCP was higher in the 43 patients infected through blood transfusion than in the 25 intravenous drug users (mean number of bands: 5.8 ± 2.3 vs. 4.7 ± 1.5, respectively; $P = 0.04$). It is noteworthy that genetic complexity and its relationship with the route of HCV transmission were independent of both age and putative disease duration.

In contrast, no significant relationship was found between HVR1 major variant complexity and the virological parameters of infection (HCV load and HCV genotype), markers of the immune response to HCV (anti-HCV core IgM antibodies) and the characteristics and severity of HCV liver disease (serum ALT and γ-GT activities and presence of cirrhosis on liver biopsy).

Relationship between the Genetic Complexity of HVR1 Major Variants and Responses to Interferon Alfa Therapy

Among the 101 patients in whom the genetic complexity of HVR1 could be evaluated, 43 (43%) had nor-

TABLE I. Relationship Between HVR1 Genetic Complexity of the Major Variants of HCV Quasispecies and Epidemiological, Clinical, Histological and Virological Characteristics of HCV Disease in 101 Patients with Chronic Hepatitis C

Analyzed parameters	Number of patients	Genetic complexity (number of bands in RT-PCR SSCP)
Gender		
Males	66	5.3 ± 2.1
Females	35	5.5 ± 1.9
Age		
≤35 years	32	4.8 ± 1.4
35–55 years	38	5.5 ± 2.0
>55 years	31	5.7 ± 2.5
Route of transmission[a]		
Blood transfusion	43	5.7 ± 2.3
Intravenous drug use	25	4.7 ± 1.5[b]
Disease duration[a]		
≤10 years	32	5.5 ± 1.7
>10 years	36	5.3 ± 2.4
ALT		
≤2 N	28	5.1 ± 2.4
2–3 N	25	5.6 ± 1.7
>3 N	48	5.3 ± 2.0
γ-GT		
Normal	65	5.4 ± 2.0
Elevated	36	5.3 ± 2.1
Liver biopsy		
Chronic active hepatitis	83	5.4 ± 2.1
Cirrhosis	18	5.0 ± 1.6
Viral burden		
≤4 MEq/ml	55	5.1 ± 2.0
4–8 MEq/ml	21	6.2 ± 2.2
>8 MEq/ml	25	5.1 ± 1.9
HCV genotype		
1a	21	5.0 ± 1.3
1b	44	5.9 ± 2.4
3	21	5.0 ± 1.8
Others	15	4.6 ± 1.9
Anti-HCV core IgM index		
≤100	66	5.4 ± 1.8
>100	35	5.2 ± 2.4

[a]68 patients with an identified risk factor for parenteral viral infection.
[b]$P = 0.04$.

mal ALT at month 3 of IFN treatment, 28 (28%) had normal ALT at month 6, i.e. when treatment was withdrawn from all patients, and 6 (6%) had a sustained virological response characterized by normal ALT and HCV RNA PCR negativity 6 months after IFN withdrawal, i.e. at month 12.

The genetic complexity of HVR1 major variants did not appear to affect the rate of ALT normalization at month 3 (5.1 ± 1.9 vs. 5.6 ± 2.1 bands in responders and non responders, respectively; NS) or month 6 (4.8 ± 1.7 vs. 5.5 ± 2.1 bands, respectively; NS). In contrast, there was a strong tendency towards lower HVR1 genetic complexity in patients with a sustained virological response than in non responders (4.0 ± 1.7 vs. 5.4 ± 2.0, respectively; $P = 0.07$). Two other pretreatment parameters were found to be associated with a sustained virological response in univariate analysis: a low viral load ($P < 0.01$) and a low anti-HCV core IgM index ($P < 0.07$).

TABLE II. Independent Predictors of a Sustained Virological Response to IFN Alfa Therapy (Characterized by Normal ALT and Negative HCV RNA Detection by PCR 6 Months After Treatment Withdrawal) in a Multivariate Analysis of 101 Treated Patients With Chronic Hepatitis C

Independent predictors of a sustained virological response	P value	β coefficient of the model
Low viral load (Quantiplex™ HCV RNA 2.0, Chiron)	<0.04	0.23
Low anti-HCV core IgM index (HCV IgM EIA 2.0, Abbott)	0.03	0.59
Low genetic complexity of HVR1 major variants (PCR-SSCP)	<0.04	0.02

These three parameters were entered in a multivariate analysis of pretreatment variables associated with a sustained virological response to IFN alfa. All three parameters emerged as independent predictors of a sustained virological response to IFN alfa, i.e. a low viral load ($P < 0.04$), a low anti-HCV core IgM index ($P = 0.03$) and low genetic complexity of HVR1 major variants ($P < 0.04$) (Table II).

DISCUSSION

Many attempts have been made to establish correlations between HCV RNA genetic heterogeneity and the characteristics of HCV infections, but published results are conflicting. This seems to be due mainly to inadequate definition of the parameters investigated and to the use of various techniques, whose sensitivity for quasispecies analysis had not been clearly determined in most instances. The reference method is cloning of PCR fragments followed by sequence analysis of a sufficient number of clones, i.e. more than 20 [Gretch and Polyak, 1997]. However, cloning and sequencing is labour-intensive and expensive, and cannot be used easily for establishing clinical correlations in large series of patients. Several rapid methods have been developed for studying HCV genetic heterogeneity, such as direct sequencing and polymorphism analysis [Odeberg et al., 1995], heteroduplex gel shift analysis [Wilson et al., 1995], heteroduplex analysis by temperature gradient gel electrophoresis (TGGE) [Lu et al., 1995] and SSCP [Enomoto et al., 1994; Gonzalez-Peralta et al., 1996; Koizumi et al., 1995; Moribe et al., 1995; Sakamoto et al., 1995].

To ensure standardization, sensitivity and reproducibility, we developed a PCR-SSCP procedure for HCV HVR1 genetic heterogeneity based on the use of highly standardized (commercially available) reagents and materials, in which the number of bands was objectively calculated by computer. The technique appears to have better sensitivity than previously published "in-house" SSCP methods [Enomoto et al., 1994; Koizumi et al., 1995; Moribe et al., 1995]. Indeed, it detected a higher number of bands in patient sera, as was the case in another study in which commercial reagents and materials were also used [Gonzalez-Peralta et al., 1996]. In addition, our technique is capable of discriminating variants differing by point mutations,

and is highly reproducible when different extraction products of the same serum are used (data not shown). Based on experiments with sequenced clones, it was shown that the method can detect variant populations representing as little as 10% of the entire quasispecies. The technique is therefore a reliable tool for evaluating genetic complexity of HVR1 "major variants".

In this study it was found that the genetic complexity of HVR1 major variants was not associated with any other parameter of HCV infection or liver disease. Associations between HCV genetic heterogeneity and parameters of infection, such as HCV genotype, viral load or disease duration, have been reported occasionally. Discrepancies in the literature, together with the lack of any significant relationship between HVR1 major variant genetic complexity and virological parameters of infection (viral load and HCV genotype) in the present study where a high number of patients were analyzed, suggest that the relationship between quasispecies heterogeneity and the characteristics of infection is more complex than expected. Indeed, it was thought that high viral loads or particular genotypes could generate higher mutation rates and, thus, higher HCV genome complexities [Gonzalez–Peralta et al., 1996]. It must be emphasized, however, that the region studied (HVR1) is the target of neutralizing immune responses [Weiner et al., 1992; Farci et al., 1994]. The emergence and development of quasispecies in this region is therefore the result of complex interactions between: (i) specific characteristics, including genetic heterogeneity, of the infecting strain, and (ii) the host immune response, which is genetically determined and can be subject to various external factors. A possible influence of viral strain characteristics at the time of infection on later genetic heterogeneity may not thus have been visible at the time of the study.

In contrast, the genetic complexity of HVR1 major variants was significantly higher in transfusion recipients than in intravenous drug users. Interestingly, this relationship was independent of disease duration and age, although drug users were younger and had been infected more recently. Apart from age and disease duration, the main difference between transfusion recipients and intravenous drug users is the larger initial viral inoculum in the former. It is likely that the "big bang", which results from the initial interaction between the viral inoculum and the genetic bottleneck and leads to the emergence and development of the viral quasispecies, is influenced by the amount and, possibly, the complexity and diversity of the initial HCV populations. It is thus conceivable that large and complex inocula, characteristic of transmission by transfusion, lead to more complex HVR1 quasispecies populations. It must be stressed, however, that this hypothesis cannot be tested retrospectively. Differences in host immunological response could also conceivably explain changes in genomic complexity observed in different groups of patients.

A major finding was the independent predictive role of HVR1 major variant genetic complexity on the sustained virological response to IFN alfa. Preliminary results, based on small series of patients and univariate analyses, also suggested a predictive influence of HCV genetic heterogeneity on biochemical responses to IFN [Gonzalez-Peralta et al., 1996; Kanazawa et al., 1994; Koizumi et al., 1995; Moribe et al., 1995; Okada et al., 1992; Shindo et al., 1996]. In the present study, a larger number of patients were examined. The predictive role of genetic complexity on viral clearance was studied and a sufficient number of parameters in analysis included to permit multivariate analysis. Using this procedure, three independent predictors of a sustained virological response to IFN therapy were identified, including a low viral load, a low anti-HCV core IgM titer and low HVR1 major variant genetic complexity. The usually low rates of response to IFN could be explained by two, mutually compatible, hypotheses : (i) patients could be naturally resistant to IFN, and (ii) infecting viruses could be naturally resistant to IFN. As far as the first hypothesis is concerned, it was shown recently that non-responders had normal signal transduction pathways required for the intracellular activity of IFN. Indeed, the response to IFN was not related to the activity or inducibility of the intracellular enzyme 2′-5′ oligoadenylate synthetase, which partly mediates the effect of IFN on target cells [Pawlotsky et al., 1996a]. Conversely, several studies, including ours, suggested a major role of viral parameters, e.g. viral load and HCV genotype, in the responses to IFN therapy [Mahaney et al., 1994; Martinot-Peignoux et al., 1995; Pawlotsky et al., 1996b]. The results of the present study, showing an independent predictive influence of viral load, the anti-HCV core IgM index and the genetic complexity of HVR1 major variants on the sustained virological response to therapy, suggest that the main factor determining whether HCV infection is sensitive or resistant to IFN treatment is the state of the interaction between the virus and host at the time of therapy. Indeed, all three parameters are indirect markers of this state: viral load likely depends on the control of viral replication by neutralizing and cytotoxic responses, the IgM index is a marker of the host immune response, the exact significance of which remains unclear, and HVR1 major variant genetic complexity reflects neutralizing immune pressure on virus replication [Weiner et al., 1992; Farci et al., 1994; Gretch and Polyak, 1997; Kumar et al., 1994]. The sensitivity of chronic hepatitis C to IFN could therefore depend on the capacity of the host/virus equilibrium to be tipped by IFN in favor of viral clearance. Additional data on genetic heterogeneity in regions of the genome targeted by different selective pressures will, however, be necessary to understand the relationship between HCV genetic heterogeneity and the response to IFN therapy.

In summary, the results show a significant association between HVR1 major variant genetic complexity and the route of HCV transmission, suggesting that the size and complexity of the viral inoculum at the time of infection influence the later evolution of viral quasispe-

cies in a given patient. In addition, low HVR1 major variant genetic complexity was shown to be an independent predictor of sustained responses to IFN therapy, together with a low viral load and a low anti-HCV core IgM index. The role of minor variants and genetic diversity in HCV-induced liver disease and the response to IFN treatment remains to be determined.

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